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Research Note

Effect of Freezing, Irradiation, and Frozen Storage on Survival of *Salmonella* in Concentrated Orange Juice†

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ABSTRACT

Six strains of *Salmonella* (Anatum F4317, Dublin 15480, Enteritidis 13076, Enteritidis WY15159, Stanley H0588, and Typhimurium 14028) were individually inoculated into orange juice concentrate (OJC) and frozen to −20°C. The frozen samples were treated with 0 (nonirradiated), 0.5, 1.0, or 2.0 kGy of gamma radiation and held frozen for 1 h, and the surviving bacterial population was assessed. The strains showed significant variability in their response to freezing and to freezing in combination with irradiation. The response was dose dependent. Relative to the nonfrozen, nonirradiated control, the reduction following the highest dose (2.0 kGy) ranged from 1.29 log CFU/ml (*Salmonella* Typhimurium) to 2.17 log CFU/ml (*Salmonella* Stanley). Samples of OJC inoculated with *Salmonella* Enteritidis WY15159 and irradiated were stored at −20°C for 1, 2, 7, or 14 days, and the surviving population was determined. Relative to the nonfrozen, nonirradiated control, after 14 days, the population was reduced by 1.2 log CFU/ml in the nonirradiated samples and by 3.3 log CFU/ml following treatment with 2.0 kGy. The combination of frozen storage plus irradiation resulted in greater overall reductions than either process alone.

Freezing is known to reduce the viability of pathogens such as *Salmonella, Escherichia coli* O157:H7 and *Listeria monocytogenes,* typically in a strain-dependent manner *(4, 11).* Relatively brief periods (e.g., 1 h) of frozen storage can reduce viable cell counts by 1 to 2 log units, with extended storage causing additional, time-dependent reductions *(14).* However, high levels of dissolved sugars tend to have a protective effect on suspended bacteria in frozen solutions, reducing the antimicrobial effect *(2, 14).* The composition of the food medium has a direct influence on the survival and injury level of the frozen bacterial population *(2, 5, 9).*

Ionizing radiation has been suggested as an alternative to thermal pasteurization to achieve the recommended 5 log reduction of pathogens in fresh juices *(7).* Irradiation effectively eliminates bacteria from fresh fruit juices, with radiation sensitivity influenced by bacterial strain and, in some cases, juice composition *(1, 3, 8, 10).* The effects of irradiation on food sensory characteristics are typically diminished when the process is conducted at subfreezing temperatures, but these lower temperatures typically increase bacterial radiation resistance *(9, 12, 13).* The possibility of combining the antimicrobial effects of irradiation and freezing and frozen storage has shown promise with chicken broiler carcasses *(6),* but comparable studies on frozen juice products, particularly those with high osmotic strength such as juice concentrates, are lacking.

The objectives of this study were to determine (i) the radiation sensitivity of six *Salmonella* isolates in frozen orange juice concentrate (OJC) and (ii) the combined effect of irradiation and frozen storage on an orange juice outbreak-associated strain of *Salmonella* Enteritidis.

MATERIALS AND METHODS

Microorganism. Six serovars of *Salmonella* were used for these experiments: *Salmonella* Dublin 15480, *Salmonella* Enteritidis 13076, *Salmonella* Typhimurium 14028 (American Type Culture Collection, Manassas, Va.); *Salmonella* Anatum F4317, *Salmonella* Stanley H0588 (Centers for Disease Control and Prevention, Atlanta, Ga.); and *Salmonella* Enteritidis WY15159 (Wyoming Department of Health, Cheyenne, Wyo.). *Salmonella* Enteritidis WY15159 is a strain of *Salmonella* Enteritidis isolated from salmonellosis patients who consumed contaminated, unpasteurized orange juice. Stock cultures were maintained in tryptic soy broth (Difco, Sparks, Md.) at 2°C and transferred bimonthly.

Inoculation. Commercially available frozen OJC (retail concentrate) was purchased from local markets and stored at −70°C until used. The frozen OJC was thawed in a refrigerator (2°C) overnight before each experiment.

Working cultures were grown by inoculation of 10 ml of sterile tryptic soy broth with 0.1 ml of stock culture and incubation at 37°C for 18 h with agitation. The concentration of each working culture was determined by dilution with Butterfield's phosphate buffer (0.25 M KH_2PO_4, adjusted to pH 7.2 with NaOH) (Applied Research Institute, Newtown, Conn.), pour plating using tryptic soy agar (TSA; Difco), and incubation for 24 h at 37°C. The concentrations were determined to be approximately 10^9 CFU/ml. Acid adaptation can reduce the radiation sensitivity of suspended bacteria *(1).* In order to better isolate the effects of cryotolerance and radiation sensitivity, the cultures were not acid-adapted before inoculation. Aliquots of working culture (0.4 ml

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† Mention of brand or firm names does not constitute an endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

per 10 ml OJC to be inoculated) were centrifuged at 5,000 × *g* for 10 min to pelletize the cells. The tryptic soy broth supernatant was discarded, and thawed OJC was added to the pelletized cells. The thawed OJC was vortexed until the pellet was visibly dispersed, to resuspend the cells. The final bacterial concentration in the inoculated OJC preparation was approximately 10^8 CFU/ml. The nonfrozen, nonirradiated inoculated OJC was used as the control. A 1-ml sample of the control OJC was serially diluted with Butterfield's phosphate buffer, pour plated using TSA, and incubated at 37°C for 24 h.

For the *Salmonella* isolate comparison study, aliquots (5 ml) of individually inoculated OJC were dispensed into separate polypropylene tubes, one per dose, placed in a freezer (−20°C) prior to irradiation, and allowed to freeze by contact with the chilled air. Samples were frozen solid within 15 min.

For the frozen storage study, OJC was inoculated with *Salmonella* Enteritidis WY15159, and aliquots were dispensed into individual tubes as described, one tube per dose/sampling time combination.

Irradiation. Samples for the isolate comparison study and the frozen storage study were irradiated using the same protocol. Each study was performed three times. The samples were held at −20°C before, during, and after irradiation. Temperature control was maintained during irradiation by thermocouple-controlled injection of gas-phase liquid nitrogen into the sample chamber. Samples were treated with 0 (nonirradiated), 0.5, 1.0, or 2.0 kGy. The samples were irradiated using a Lockheed-Georgia (Marietta, Ga.) cesium-137 self-contained gamma radiation source, with a dose rate of 0.098 kGy/min. The dose rate was established using alanine transfer dosimeters from the National Institutes of Standards and Technology (Gaithersburg, Md.). Alanine pellets (Bruker, Inc. Billarica, Mass.) were used for dosimetry. The pellets were read on a Bruker EMS 104 EPR analyzer and compared with a previously determined standard curve. Actual dose was typically within 5% of the nominal dose.

Storage and sampling: isolate comparison. For the isolate comparison study, the samples were taken from the freezer shortly after irradiation of the final sample. Total time frozen for all samples was approximately 1 h. Irradiated and nonirradiated samples were allowed to thaw in air to room temperature approximately 30 min. The thawed samples were vortexed and a 1-ml aliquot was removed and serially diluted with Butterfield's phosphate buffer. Dilutions ranging from 10^3 to 10^6 were pour-plated using TSA, with three plates (subsamples) per dose for each isolate. The time between dilution and subsequent plating was approximately 30 to 60 min. The TSA plates were incubated at 37°C for 24 h. The plates were counted with an AccuCount 1000 automated counter (Biologics, Gainesville, Va.). The populations were expressed as absolute log CFU/ml and as reductions from the nonfrozen, nonirradiated control. The experiment was conducted three times, the data were pooled, and the final population was $n = 9$ per dose/isolate combination. For each isolate, the reduction from the nonfrozen, nonirradiated control at each dose was evaluated with ANOVA ($P < 0.05$). The reductions are scaled to adjust for differences in initial populations among the isolates. Similarly, for each dose, the differences in reduction from control among the isolates were evaluated with analysis of variance ($P < 0.05$).

Storage and sampling: frozen storage. For the frozen storage study, the samples were held at −20°C and sampled after 1, 2, 7, and 14 days. Irradiated and nonirradiated samples were allowed to thaw in air to room temperature. The thawed samples were vortexed and a 1-ml aliquot was removed and serially diluted with Butterfield's phosphate buffer. Dilutions ranging from 10^3 to 10^6 were pour-plated using TSA, three plates per dose/time combination. The TSA plates were incubated at 37°C for 24 h. The plates were counted with an AccuCount 1000 automated counter (Biologics). The populations were expressed as reductions from the nonfrozen, nonirradiated control. The experiment was conducted three times, and the data were pooled; the final population was $n = 9$ per dose/time combination. For each sampling time, differences between doses were evaluated with ANOVA ($P < 0.05$).

RESULTS

Isolate comparison. For all isolates, irradiation reduced the bacterial population in a dose-dependent manner (Fig. 1). In the absence of irradiation, a brief period of freezing did not cause significant changes in the population relative to the control in five of the six isolates examined (Table 1). For *Salmonella* Enteritidis WY15159, the bacterial counts in the frozen, nonirradiated samples were 0.24 log CFU/ml greater than in the control. *Salmonella* Typhimurium was generally the least sensitive to radiation, whereas *Salmonella* Enteritidis 13076 was generally the most sensitive. However, each isolate displayed a unique pattern of response to the various levels of irradiation. Reductions ranged from 0.46 to 1.17 log CFU/ml following 0.5 kGy, 0.86 to 1.46 log CFU/ml following 1.0 kGy, and 1.29 to 2.17 log CFU/ml following 2.0 kGy (Table 1).

Frozen storage. The population of *Salmonella* Enteritidis WY15159 declined during frozen storage following all levels of irradiation (Fig. 2). Higher radiation doses resulted in greater overall reductions in population relative to the control at each sampling time. Increasing time in storage caused significant ($P < 0.05$) additional reductions in population for all doses.

DISCUSSION

The antimicrobial efficacy of ionizing radiation is influenced by product composition, processing conditions, and the sensitivity of the target organism. In this study, irradiation reduced the populations of six *Salmonella* isolates in frozen OJC, but each isolate displayed a unique response pattern to the various doses examined. These results arise from the combined treatments of the transient period in thawed OJC, freezing pre- and postirradiation, and the osmotic shock associated with thawing. The differential response of the six isolates under identical experimental conditions confirm previously demonstrated isolate-level variability in radiation sensitivity for pathogens treated in juice products *(1, 10)*. Application of irradiation to frozen foods is generally regarded as advantageous because product quality following irradiation is often better maintained when products are irradiated at subfreezing temperatures *(13)*.

In the absence of irradiation, a relatively brief freezing treatment did not significantly reduce the population of any isolate relative to the nonfrozen, nonirradiated control, suggesting that the osmotically strong OJC served to protect the bacteria from the antimicrobial effects typically observed in brief freezing treatments *(2, 14)*. An isolate pre-

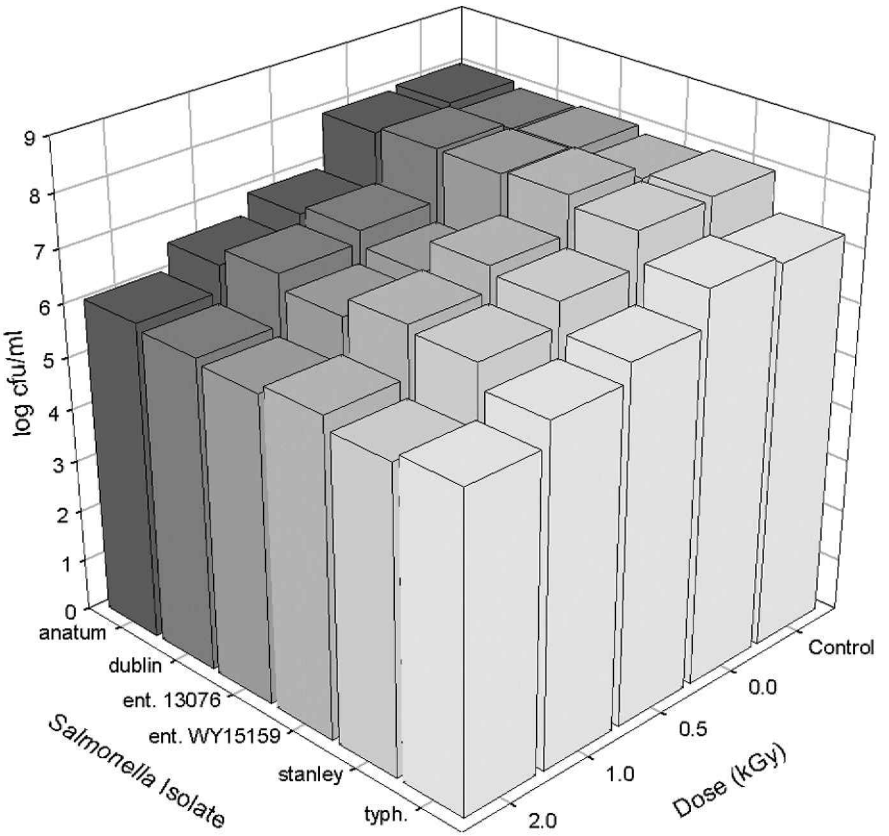

FIGURE 1. *Population (CFU/ml) of* Salmonella *Anatum F4317,* Salmonella *Dublin 15480,* Salmonella *Enteritidis 13076,* Salmonella *Enteritidis WY15159,* Salmonella *Stanley H0588, and* Salmonella *Typhimurium 14028 in irradiated frozen concentrated orange juice and in a nonfrozen, nonirradiated control.* n = 9 *per isolate/dose combination.*

viously associated with an orange juice outbreak, *Salmonella* Enteritidis WY15159, showed a slight (0.24 log CFU/ml) but statistically significant increase in recoverable bacteria following the brief freezing treatment. The extent of the increase suggests that the practical significance of this result is minimal; however, this phenomenon may serve as the basis for future research to eliminate the possibility that it is artifactual in nature.

In this study, *Salmonella* Enteritidis WY15159 declined significantly in frozen samples during the course of 14 days of frozen storage. This pattern was consistent for all radiation treatment levels. At each sampling time, the population in samples treated with 1.0 or 2.0 kGy was significantly less than in the nonirradiated samples. Notably, however, the recoverable populations in samples treated with a relatively low dose, 0.5 kGy, were more intermediate. This is in contrast to the clear statistical separation observed after the brief freezing treatment. Freezing and cold shock can sensitize bacteria to subsequent stresses *(2),* and it has been suggested that transient storage in nonfrozen juice might similarly sensitize bacteria to subsequent frozen storage *(14).* The combination of low-dose irradiation with other antimicrobial processes to achieve target reductions in bacterial load has previously been examined *(1, 6). Enterobacteriaceae* and *Salmonellae* were effectively eliminated from chicken broiler carcasses by a combination of irradiation and frozen storage, a combination of processes that was more effective than freezing alone *(6).*

In this study, irradiation and frozen storage was more effective than either treatment alone at eliminating *Salmonella* from OJC. After the brief freezing period, and at each sampling time during frozen storage, samples treated with

TABLE 1. *Reduction of the population of* Salmonella *isolates in irradiated frozen concentrated orange juice*

	Salmonella isolate reduction[a,b]											
	Anatum F4317		Dublin 15480		Enteritidis 13076		Enteritidis WY15159		Stanley H0588		Typhimurium 14028	
Dose (kGy)	CFU/ml	*P*	CFU/ml	*P*	CFU/ml	*P*	CFU/ml	*P*	CFU/ml	*P*	CFU/ml	*P*
Control	0.00	A	0.00	A	0.00	A	0.00	A	0.00	A	0.00	A
0.0	−0.07 H	A	0.13 FG	A	0.06 GH	A	0.24 F	B	−0.06 H	A	0.14 FG	A
0.5	−1.07 G	B	−0.83 G	B	−1.17 H	B	−0.46 F	C	−0.71 G	B	−0.53 F	B
1.0	−1.46 I	C	−1.06 GH	C	−1.38 I	C	−0.89 FG	D	−1.13 H	C	−0.86 F	C
2.0	−1.95 GH	D	−1.98 HI	D	−2.12 IJ	D	−1.81 G	E	−2.17 J	D	−1.29 F	D

[a] Reductions are expressed as log CFU/ml relative to the nonfrozen, nonirradiated control. $n = 9$ per dose/isolate combination.

[b] Within each *Salmonella* isolate, values for a given dose followed by a different letter are significantly ($P < 0.05$) different (ANOVA). Within each dose, values for a given *Salmonella* isolate followed by a different letter are significantly ($P < 0.05$) different (ANOVA).

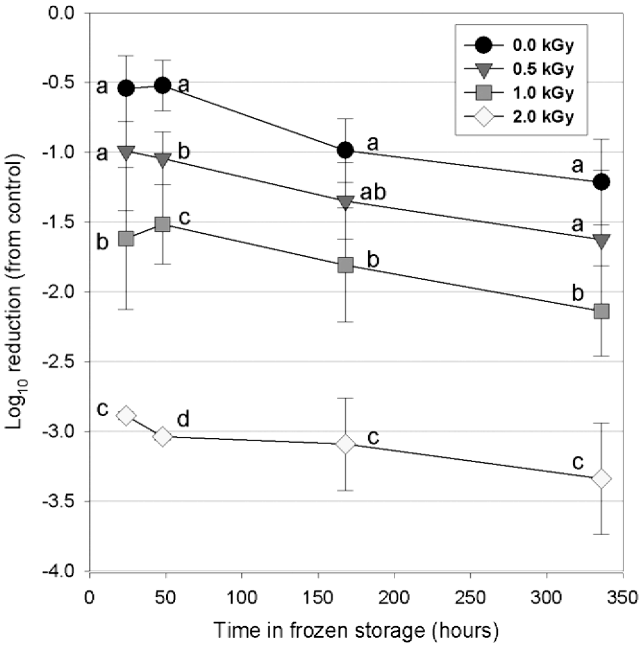

FIGURE 2. *Survival of* Salmonella *Enteritidis WY15159 in frozen concentrated orange juice following irradiation and frozen storage. Within each sampling time, values with different letters are significantly* ($P < 0.05$) *different from each other (ANOVA). Bars indicate standard error,* $n = 9$.

the 2-kGy dose showed approximately 2.0 log CFU/ml fewer recoverable bacteria than the nonirradiated control. Therefore, the action of the combined treatments appeared to be additive, rather than synergistic, in nature for this product.

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